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Effect of toyocamycin on oncornaviral production by acutely infected cells.

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Résumé.

La toyocamycine est un analogue de l'adénosine qui s'incorpore dans toutes les espèces de RNA dans les cellules de mammifères. A faibles concentrations l'analogue abolit l'apparition des RNA ribosomiques dans le cytoplasme, par blocage de maturation du RNA préribosomique nucléolaire 45S. Ces faibles concentrations inhibent totalement la production virale dans un système cellulaire primo-infecté par le complexe viral de Friend ; elles n'inhibent cependant ni la synthèse, ni la maturation par épissure des espèces intracellulaires virales qui peuvent être détectées dans les expériences d'hybridation sur répliques avec les sondes provirales spécifiques. Les messagers viraux intracellulaires synthétisés semblent être traduits correctement en leurs protéines correspondantes.

Ainsi, les maturations des RNA ribosomiques, d'une part, et des RNA messagers viraux, d'autre part, procèdent de mécanismes différents, bien que les structures secondaires de ces macromolécules soient impliquées dans les deux cas ; de plus, la présence de résidus toyocamycine incorporés dans les messagers viraux n'empêche pas la traduction en protéines virales spécifiques. L'inhibition de la production virale semble donc s'exercer à une étape ultérieure qui peut se situer au niveau de l'assemblage des particules virales.

Ces résultats, obtenus avec un système primo-infecté, confirment ceux publiés précédemment utilisant un système producteur chroniquement infecté et lèvent toute ambiguité quant au rôle éventuel des RNA messagers spécifiquement viraux préexistants et demeurant fonctionnels après l'adjonction de l'analogue.

Mots-clés : toyocamycine / virus de Friend / primo-infection / RNA viraux intracellulaires.

Summary.

The adenosine analogue toyocamycin incorporates into the RNA species of mammalian cells and abolishes at low concentrations the processing of 45S preribosomal nucleolar RNA into the mature 28 and 18S cytoplasmic ribosomal RNAs. We have previously shown that toyocamycin depresses the production of the Friend leukemia viral complex by chronically infected cells.

In this article, we report the study of the action of the drug on viral RNA in acutely infected cells. We found that, although abolishing viral production, the incorporation of toyocamycin does not inhibit the formation of mature viral messenger RNAs nor prevent the synthesis of specific viral proteins. These results are obtained at concentrations of analogue sufficient to abolish the appearance of mature cytoplasmic ribosomal RNA.

Key-words : toyocamycin / Friend viral complex / acute infection / intracellular viral RNAs.

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Introduction.

Low concentrations of the adenosine analogue toyocamycin (4-amino-5-cyano-7-β-D-ribofuranosyl-pyrrolo 2,3-d pyrimidine) selectively abolish the appearance of newly synthetized 28S and 18S ribosomal RNA (rRNA) in the cytoplasm of mammalian cells, but allow the synthesis of other RNA species (transfer RNA, 5S RNA, rapidly labelled nuclear RNAs), including the 45S ribosomal RNA precursor which accumulates in the cell's nucleoli. The 45S RNA containing toyocamycin is normally methylated but cannot be converted into 28S and 18S rRNAs after removal of the analogue from the medium, whereas the normally pulse-labelled 45S RNA is converted into rRNA even in the presence of toyocamycin [1]. These findings strongly suggest that the transcription of the various cellular RNA species on their DNA template is not affected by low concentrations of toyocamycin and that the lack of conversion of the nucleolar 45S precursor into the mature 28S and 18S RNAs is due to the alteration of specific structures constituting cleavage sites for the maturation of ribosomal RNA.

Because of this selective effect, several authors have investigated the influence of this analogue on the replication of mammalian RNA and DNA viruses. Toyocamycin inhibits the replication of adenovirus [2-3], vesicular stomatitis virus [4] and retrovirus [5-6], whereas the replication of a picornavirus, the encephalomyocarditis virus, is not affected [7].

Our group has shown that toyocamycin decreases both the number and the infectivity of retrovirus particles released by a cell line chronically infected by the Friend leukemia virus : the Eveline-Friend cells [6]. It was observed that tritiated toyocamycin was incorporated into the genomic RNA of the released viruses [8] and that the endogenous reverse transcriptase activity of these virions was greatly diminished, although exogenous activity, using either poly rA-oligo dT or poly rC-oligo dG, was identical whether the enzyme originated from toyocamycin-treated or normal virions. It was therefore suggested that the enzyme was not modified but could not correctly transcribe the viral genome whose structure was altered by the presence of the analogue.

However the results obtained with the Eveline-Friend cell line were unconclusive since virus production is permanent in chronically infected cells ; it could well be that the viral messenger RNAs synthesized prior to the addition of the analogue could remain functional for a certain period of time thereafter [9]. Therefore, a cell system acutely infected with the Friend viral complex appeared to be more appropriate for studying the effect of toyocamycin.

The Friend murine leukemia virus complex contains a replication-defective spleen focus-forming virus (F-SFFV) and a non-defective Friend murine leukemia virus (F-MuLV). Replication of both viral genomes (respectively 32S and 38S in size) requires the synthesis of double-stranded DNA intermediates which are synthesized on the viral RNA genomes by reverse transcription and are subsequently integrated as proviruses in the host cellular DNA. The proviruses are complete transcriptional units and are transcribed like cellular genes. The non defective F-MuLV virus has three genes : *gag*, which codes for a precursor cleaved to yield four internal structural proteins of the virions, *pol*, which codes for the reverse transcriptase, and *env*, which codes for the virion envelope glycoproteins. It is assumed that these three genes code for all the information necessary for the replication of both F-MuLV and F-SFFV. Several species of viral messenger RNAs can be found in cells acutely infected with the viral complex. The mRNAs for the *gag* polyprotein and the *pol* precursor are inseparable from each other and from the total genome. The mRNA for *env* is a portion of the total genome consisting of all the information up to the 3' end (24S in size). This messenger is processed from the entire genome by splicing and contains a segment at its 5' end which is derived from the 5' end of the genome [10-11]. In addition, another spliced mRNA (20S) coding for a glycoprotein, not exported to the virions but related to the *env*. glycoprotein, is encoded by the SFFV genome and processed in a manner similar to the 24S mRNA [12, 13].

The studies reported in this work on a cell system acutely infected with the Friend virus complex were aimed at investigating whether or not toyocamycin inhibits the maturation and/or the translational activity of the viral messenger RNAs.

Materials and Methods.

Cell cultures and virus infection.

The Friend leukemic cells used in the present studies (strain 745 A) were donated by H. Tapiero and propagated in suspension culture in Joklik's modified medium supplemented with 10 per cent fetal calf serum. The viral complex produced by these cells was chosen as the virus source for infection ; it has an excess of F-SFFV over the non-defective helper F-MuLV and is much more

infectious than the complex produced by the Eveline-Friend cell line we used in former studies.

The 3T3 Swiss murine D55 strain was used as the target cell line for virus infection. The cells were propagated as monolayers in modified Eagle's medium supplemented with 10 per cent heat-inactivated fetal calf serum. 24 hours before infection 10 μg/ml of polybrene were added to the exponentially growing cells.

The cells were infected by being exposed for 4 × 15 min incubation periods to the cell free supernatant collected from the 745-A Friend leukemic cell line.

RNA separation and hybridization.

Total or (when separated) cytoplasmic and nuclear RNAs were extracted by a sodium dodecyl sulfate-phenol treatment, as previously described [14]. Poly A-containing RNAs were selected by an oligo-dT cellulose column [15].

RNAs were denatured by heating 30 minutes at 50°C in 50 per cent dimethyl sulfoxide containing 1 M glyoxal and 10 mM sodium phosphate buffer, pH 7 [16]. They were then subjected to electrophoresis in 1 per cent agarose gel in 10 mM sodium phosphate and transfered onto activated diazobenzyloxymethyl paper [17]. [^{32}P] viral cDNA was prepared with AMV polymerase (obtained from the National Cancer Institute) and salmon sperm DNA primer [18]. The specific activity of the labelled [α^{32}P] dCTP (New England Nuclear) was 600 Ci/mM. Hybridization was performed at least 24 hours at 42°C in the presence of sodium dextran sulfate [19]. After extensive washing, specific RNAs were revealed by autoradiography against Royal X-O. Mat Kodak films.

Immunoprecipitations.

Control cells and cells treated with toyocamycin were incubated one hour at 37°C in a leucine- and valine-free medium. 150 μCi/ml of tritiated valine and 150 μCi/ml of tritiated leucine (Commissariat à l'Energie Atomique, Saclay, France — specific activities 13 Ci/mM and 5 Ci/mM, respectively) were added for 20 min. The medium was then washed away and replaced with complete medium for one additional hour at 37°C. Cellular extracts were prepared as reported previously [20]. Anti-gp70 and anti-p30 antisera (obtained from the National Cancer Institute) were added to the cell lysate and incubated two hours at + 4°C. Antigen-antibody complexes were precipitated by the addition of *Staphylococcus aureus* suspension, dissociated, and loaded onto a 5-20 per cent polyacrylamide slab gel [21]. Gels were treated for fluorography as described previously [22].

Results.

I. *Influence of toyocamycin on the RNA expression of Friend leukemia viral complex in acutely infected mouse fibroblasts.*

D55 mouse fibroblasts were infected at high multiplicity, as described in Methods. The time ne-

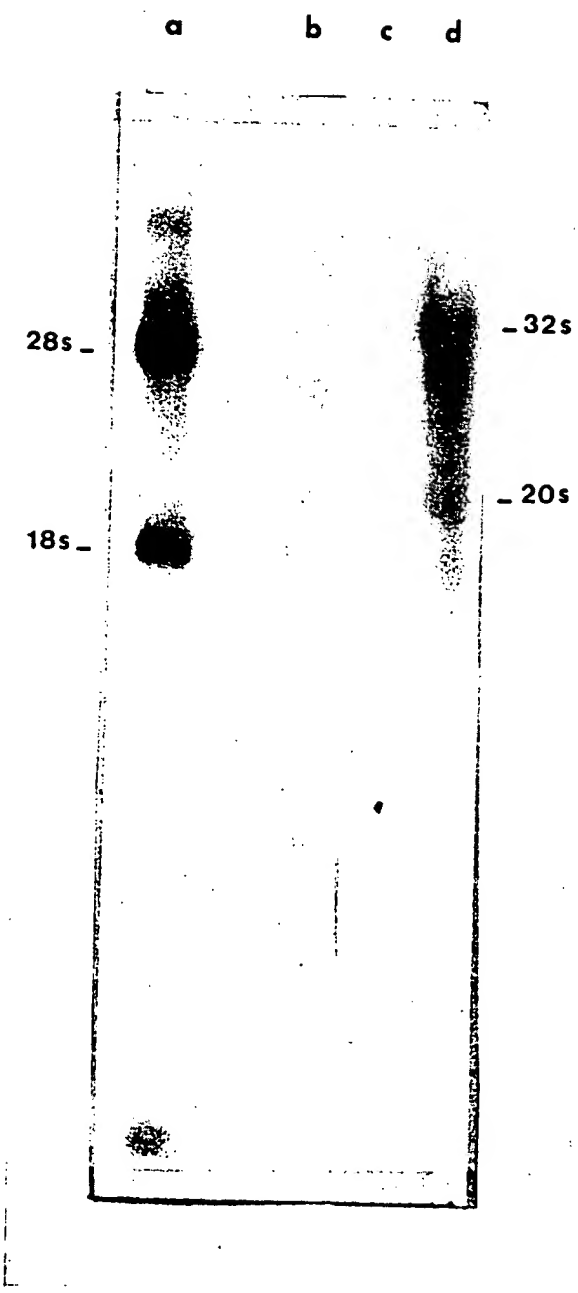

FIG. 1. — *Blotting hybridization of viral specific intracellular RNA in acutely infected cells.*

Approximately 20 μg of poly A$^+$ RNAs extracted from uninfected D55 cells (b) or infected cells 1 (c) and 3 days (d) after infection were treated with glyoxal and electrophoresed on 1 per cent agarose gels. The RNAs were blotted onto diazobenzyloxymethyl paper and hybridized with [^{32}P] viral cDNA. Lane a represents cellular [^{32}P] RNAs migrated as markers.

cessary for intracellular viral RNA detection (under our experimental conditions) was first determined in preliminary experiments. Figure 1 shows that viral RNA bands of the poly A$^+$ fraction could not be detected by hybridization 1 day after infection (lane c) but were clearly visible 3 days after infection (lane d). Several bands hydridized : the 32S RNA of the replication-defective spleen focus forming virus (SFFV) and a 20S spliced mRNA. We could never detect the intracytoplasmic 38S RNA of the helper virus (F-MuLV) in acutely infected cells : this band was seen only in well established produced cell lines. It should be stated that the Friend leukemic cell line we used for our study produces a large excess of the defective SFFV component. Beside the two 32S and 20S viral RNA species there were two other bands migrating close to the 28S and 18S ribosomal RNA markers. These bands seemed to be viral-specific since they were not visible in non-infected cells and since our

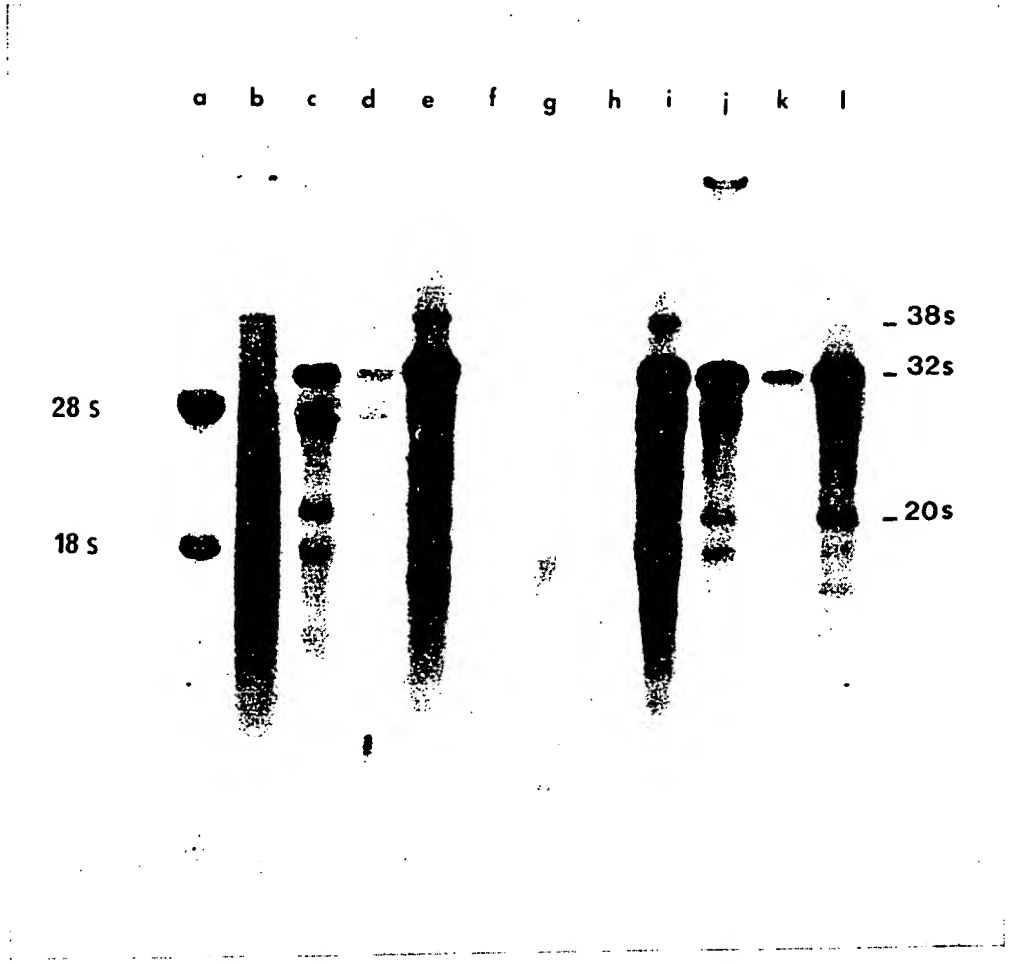

FIG. 2. — *Blotting hybridization of viral specific intracellular RNAs from toyocamycin treated and untreated acutely infected cells.*

RNAs from the various cells were processed as in fig. 1 and hybridized with viral [^{32}P] cDNA. Total [^{32}P] RNAs migrated as markers : (a), viral RNAs produced by Friend-Eveline cell line : (b). Poly A$^+$ RNAs from acutely infected D55 cells without (c) or with a 0.2 µg/ml toyocamycin treatment (d). Poly A$^-$ RNAs from acutely infected D55 cells without (g) or with 0.2 µg/ml of toyocamycin (h). Total (i) and poly A$^+$ RNAs (e) from Friend leukemic cells. Poly A$^+$ RNAs from uninfected D55 cells (f) and from acutely infected D55 cells treated with 0.2 µg/ml (j) 0.5 µg/ml (k) toyocamycin or untreated (l).

TABLE I.

Influence of toyocamycin on rAdT-dependent reverse transcriptase activity of viruses released by acutely infected D55 cells.

Concentration of toyocamycin µg/ml	cpm [H³] incorporated (X 10⁻³) Days after infection			
	0	1 (a)	2	3
0	2.7	4.0	45	65
0.2	2.7	4.0	6.0	6.0
0.5	2.7	4.0	5.0	3.7

(a) Toyocamycin was added one day after infection.

cDNA probe was synthesized on a viral RNA template selected by oligo-dT affinity chromatography and devoid of ribosomal RNAs. However comigration of partially degraded viral RNAs with some ribosomal RNAs cannot be excluded.

In order not to interfere with the early phases of infection : *i.e.*, formation of the provirus and integration of the proviral DNA in the host cellular genome, toyocamycin was added in all the following experiments 20-24 hours after infection. At that time, no specific viral RNA can be detected in the cytoplasm of infected D55 cells (fig. 1, lane c). 24 hours later (48 hours after infection), the RNAs were extracted from treated and untreated cells. As controls, the RNAs of uninfected fibroblasts and chronically infected cells were also extracted. The poly A⁺ RNAs were then selected, processed and analyzed as described above.

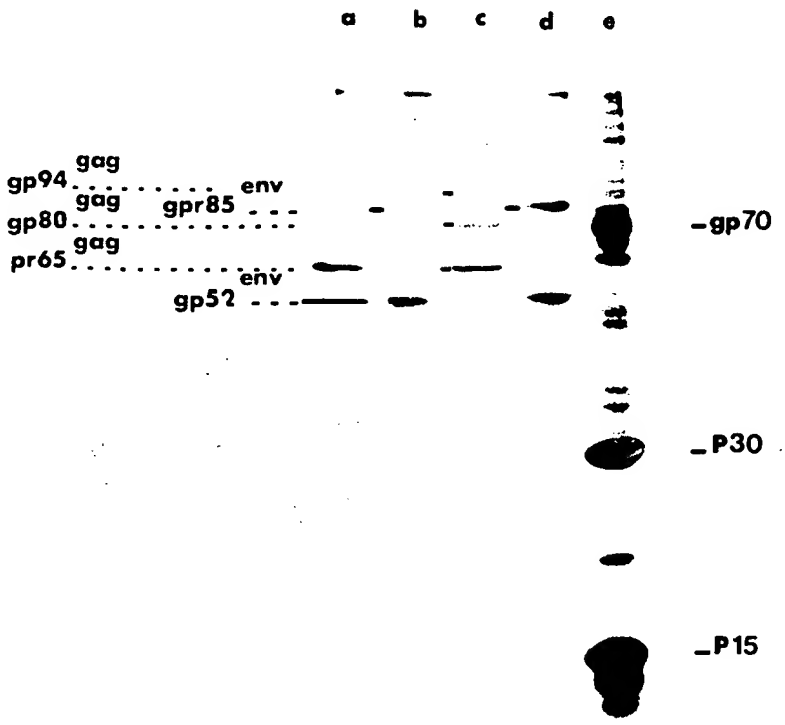

FIG. 3. — *Analysis of the viral proteins precipitated with anti-p30 (a-c) and anti-gp70 (b-d) Rauscher antisera.*

a-b : D55 acutely infected cells.
c-d : D55 acutely infected cells treated with toyocamycin (0.2 µg/ml).
e : Friend-Eveline virus total proteins migrated as markers.

Results are shown in figure 2. Lanes e and i represent the RNA extracted from Friend leukemic cells where the bands appear darker than in acutely infected cells (lanes c and d, for example). The 38S helper RNA is present and does correspond to the viral RNA prepared from another chronically infected cell line (the Friend-Eveline line) producing only the helper virus (F-MuLV) (lane b). D55 cells infected primarily with the Friend virus complex are represented in lanes c, d, g, h. The 32S and 20S SFFV specific RNAs are present in the poly A$^+$ RNAs from untreated cells (lane c) and cells treated with toyocamycin (lane d). In contrast, no specific hybridization appeared in the poly A-deprived fraction from untreated (lane g) and treated cells (lane h) indicating that all the viral-specific RNAs of toyocamycin treated cells were polyadenylated as were the viral-specific RNAs of untreated cells. In another set of experiments, D55 mouse fibroblasts were infected in the same manner and treated with different concentrations of toyocamycin. The poly-A$^+$ RNAs were extracted and equal amounts were migrated in the agarose gel shown in figure 2 ; they correspond to cells treated with 0.2 µg/ml (lane j), 0.5 µg/ml (lane k) or to untreated cells (lane l). The 32S and 20S viral bands were present in all cases ; they were less intense however in cells treated with toyocamycin, particularly with the higher dose used.

It should be stated that 48 hours after infection no reverse transcriptase activity could be detected in toyocamycin-treated cells in contrast with untreated cells, as shown in table I.

2. *Presence of viral proteins in the infected mouse fibroblasts treated with toyocamycin.*

The murine group specific viral proteins are synthesized *via* a pr65 gag polyprotein precursor which is consecutively cleaved into four internal proteins of the virion (named p15, p12, p30 and p10). It can be seen in figure 3 (lanes a and c) that the anti p30 serum revealed the pr65gag in both lysates from untreated and toyocamycin treated cells. Under our experimental conditions (20 minutes labeling followed by chase for one hour) the p30 protein itself was not detected in either lysate ; however, the gag related proteins gpr80 and gp94 which are known to be rapidly processed at the cell surface and then released in the culture fluid [23] were essentially observed in the lysate of toyocamycin treated cells.

The env-product of MuLV is a gp70 glycoprotein processed from a gpr85-env precursor. SFFV, which is the major component in our viral complex, expresses in addition a gp52-env possessing common antigenic determinants with gp70 [24]. Immunoprecipitation with the anti-gp70 serum revealed the anti gp52 in treated as well as in untreated cells (fig. 3, lanes b and d). The gp85 precursor was also present in both cell lysates but seemed more abundant in the sample from toyocamycin treated cells, suggesting a slackening in the conversion to the mature form.

The results indicate that incorporation of the analogue in viral mRNAs did not affect their translational function but could slow down the maturation processes of the *gag* and *env* gene products.

Discussion.

Low concentrations of toyocamycin selectively inhibit the maturation of ribosomal RNAs in mammalian cells. Owing to the incorporation of the analogue, the 45S ribosomal RNA precursor accumulates in the nucleoli and cannot be cleaved into mature products.

In procaryotic cells an endoribonuclease specific for double stranded RNAs, RNaseIII, is responsible for ribosomal RNAs maturation [25]. Such an enzyme has not yet been well characterized in eukaryotic cells, but *in vitro* studies showed that RNase III could convert the eukaryotic 45S RNA precursor into mature 28S and 18S ribosomal RNAs [26]. Under similar conditions the 45S RNA that accumulated after toyocamycin treatment could not be cleaved by RNase III [26]. This fact strongly suggests that the incorporation of the analogue into the 45S molecule might alter its secundary structure and change the stability of duplex sequences specifically recognized by RNase III.

The maturation of messenger RNAs from large precursor macromolecules seems to proceed through a more complex mechanism. Not only should there be a deletion of introns but the exons' ends have to be spliced [27-28]. Some small nuclear RNA species have been involved in the excision process, constituting duplex structures [29].

Toyocamycin inhibits the production of Friend viruses by primary infected mouse fibroblasts. This inhibition occurs provided the analogue is added soon enough after infection. When toyocamycin is added 20-24 hours after infection, there seems to be no interference with the first stages of infection (synthesis of cDNA and integration). Besides, syn-

thesis of viral messenger RNAs, even of those produced by splicing, is not affected. Moreover, there is synthesis of viral proteins in spite of the presence of the analogue. Toyocamycin is incorporated into all RNA species studied so far in mammalian cells and one of the questions we asked was whether incorporation of the analogue in lieu of some adenosine residues in mRNAs would bring about some misreading frame. The answer seems to be no, as least for the viral messenger RNAs we, and others, studied. We had already shown that the replication of encephalomyocarditis virus was not affected by toyocamycin [7]. Other authors also demonstrated that, in spite of the total inhition of virus release, the viral mRNA and proteins of vesicular stomatitis virus were all present in the cytoplasm of toyocamycin-treated cells [4].

Previous work on adenovirus-infected cells had shown that there was no appearance of viral mRNAs in the cytoplasm of toyocamycin-treated cell and that there was an accumulation of rapidly sedimenting nuclear viral RNAs [2]. These are the only discordant results that we known of ; we may point out that the concentrations of toyocamycin used in these studies were much higher.

Several years ago, at a time when nothing was suspected about splicing mechanisms for maturation of messenger RNA, we had already shown that under conditions where there was no synthesis of ribosomal RNA in the cytoplasm, toyocamycin did not prevent the attachment of newly synthesized messenger RNAs on the polyribosomes [30].

We cannot at present explain all the apparently discordant results obtained with toyocamycin concerning its effects on messenger RNA maturation and synthesis, and on specific protein expression. We observe all the viral messenger RNAs and the viral proteins of a retrovirus synthesized and yet there is no virus release. It may well be that some specific cellular functions such as those involved in glycosylation [31] are necessary for the assembly and budding process for retrovirus production.

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